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Molecular Detection of New Mutations, Resolution of Ambiguous Results and Complex Genetic Counseling Issues in Huntington Disease

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Huntington disease (HD) is an autosomal dominant neurodegenerative disorder caused by expansion of a variable length $(CAG)_n$ repeat in the 5′ coding region of a novel gene on chromosome 4p16.3. We provide comprehensive molecular analysis of a sporadic case of HD in which a paternally derived normal length allele expanded to an affected length allele. Linkage analysis and paternity testing confirm the paternal origin of the expansion and demonstrate that unequal crossing over during meiosis is an unlikely mechanism for de novo expansion in HD. This case identifies a complex genetic counseling issue for the families of sporadic cases since calculations of recurrence risk are not possible at this time. In addition, we describe utilization of a combination of polymerase chain reaction (PCR) based assays for examination of both the CAG repeat and an adjacent variable length CCG repeat in the *huntingtin* gene. The combination of these assays can increase the accuracy of molecular diagnosis for HD and may clarify any ambiguous results obtained during molecular testing of HD families.

KEY WORDS: Huntington disease, sporadic Huntington disease, CAG repeat, CAG expansion

INTRODUCTION

Huntington Disease (HD) is a dominantly inherited neurodegenerative disorder characterized by progressive motor disturbances, psychiatric and emotional changes, and cognitive decline [Duyao et al., 1993; HD Collaborative Group, 1993; Jankovic and Ashizawa, 1995]. The disease phenotype is associated with expansion of a tandemly repeated, unstable trinucleotide sequence, $(CAG)_n$, located in the 5′ coding region of the *huntingtin* gene on chromosome 4p16.3 [HD Collaborative Group, 1993]. Unaffected individuals carry up to 35 CAG repeats and affected individuals carry 36 or more CAG repeats [Andrew et al., 1993; Duyao et al., 1993; Hersch et al., 1994; Myers et al., 1993; Telenius et al., 1995]. Intermediate alleles (IAs), which range from the larger sized normal length alleles to the smaller sized disease length alleles (29–38 repeats), appear to carry some level of instability and have been shown, in the unaffected parents of sporadic cases of HD, to antecede the disease length alleles [De Rooij et al., 1993; Goldberg et al., 1993b, 1995; Myers et al., 1993].

Molecular genetic characterization of HD uses the polymerase chain reaction (PCR) to amplify the region containing the CAG repeat and determine the number of CAG repeat units present [Andrew et al., 1994; Duyao et al., 1993; Goldberg et al., 1993b; HD Collaborative Group, 1993; Myers et al., 1993]. Molecular genetic analysis of HD is frequently utilized by neurologists for diagnostic confirmation of individuals with classical symptoms and a family history of HD as well as a differential diagnostic tool for suspected cases [Broholm et al., 1994; Hersch et al., 1994]. In our experience as a diagnostic reference laboratory for HD, the majority of patients confirmed by molecular analysis to have a CAG repeat expansion also had a family history of HD. The case described herein was confirmed to have a de novo expansion of the CAG repeat. This case presents a complex genetic counseling issue for the families of sporadic HD patients.

There are currently three published PCR assays for molecular genetic analysis of this region of the *huntingtin* gene [Andrew et al., 1994]. One assay amplifies the CAG repeat only, one amplifies an adjacent variable

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length CCG repeat only, and one amplifies the two repeats together. For the molecular diagnosis of HD in our lab, we have implemented all three PCR assays, CAG, CCG, and CAG+CCG. The CAG repeat assay is essential for clinically relevant repeat length analysis and generally provides complete information for individuals and families. However, the CAG+CCG and CCG assays can be useful for resolving ambiguous results and for demonstrating heterozygosity in cases where an individual appears to be homozygous for a normal length allele using the CAG assay. This combination of techniques can clarify ambiguous results and rule out large expansions not detected by PCR, avoiding a Southern analysis. The utility of these assays will be discussed.

MATERIALS AND METHODS

CAG PCR

PCR amplification across the CAG repeat was performed as described [Andrew et al., 1994; Duyao et al., 1993; Goldberg et al., 1993b; HD Collaborative Group, 1993; Myers et al., 1993], except that the reaction was performed in 10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin (Cetus Buffer) with the addition of 200 μM dNTPs (Pharmacia, Piscataway, NJ), 10% DMSO, 600 μM 7-deaza-dGTP (Boehringer Mannheim, Germany), 0.33 μM primers [Andrew et al., 1994], 4 μCi α-^{32}P dCTP, 200 ng human genomic DNA, and 0.75 units Amplitaq (Perkin Elmer Corporation, Foster City, CA). Primer sequences were from Andrew et al. [1994]. Annealing was at 65°C and PCR was continued for 38 cycles. Products were visualized by electrophoresis on 6% acrylamide (19:1), 8.33 M urea gels at 55 W for 2 hours. Alleles sizes were determined using controls of known size and an M13 ladder.

Linkage Analysis

D4S127 and D4S126 were amplified as described [Baxendale et al., 1993; Beutow et al., 1991; HD Collaborative Group, 1993; Lin et al., 1991; Richards et al., 1991; Squitieri et al., 1994; Tagle et al., 1993; Taylor et al., 1992; Zuo et al., 1993], except that Cetus Buffer was used with DMSO as described above.

Paternity Testing

Paternity testing using eight polymorphic STR markers with a combined average power of exclusion of 99.39% was conducted as described [Alford et al., 1994].

CAG/CCG Allele Frequency and Distribution

Ninety-five normal length and thirty-five HD length chromosomes were analyzed for CAG and CCG repeat length. PCR for CCG alleles was performed as described [Andrew et al., 1994], except that Cetus Buffer was used with DMSO and 7-deaza-dGTP as described above. Primer sequences were from Andrew et al. [1994].

RESULTS

Detection of New Mutations in the *Huntingtin* Gene

We diagnosed HD in a 50-year-old man with no family history of the disease. The patient reported onset of symptoms at age 43 with gradual progression from occasional involuntary flexion of the wrists to choreiform movements of all extremities. At age 45, loss of balance was noted with facial muscle twitches and slurred speech. The patient reported cognitive decline and depression from age 46. Physical examination at age 50 revealed generalized chorea, hypotonia, myoclonus of the hands, dysarthria, and choreiform dyskinesia of the face and tongue. Muscle strength, sensory functions, and reflexes were normal, but coordination and gait were markedly disturbed by chorea. The patient demonstrated diminished cognitive function and depression with a mini-mental status score [Folstein et al., 1993] of 25/30. Magnetic resonance imaging (MRI) of the head revealed marked bilateral caudate atrophy. The parents of our patient are both over 73 years of age and show no evidence of chorea, dementia, or other psychiatric disturbance.

DNA from peripheral blood lymphocytes of our patient and his parents was analyzed for CAG repeat number using PCR primers that exclude the adjacent variable length CCG repeat [Andrew et al., 1994]. Our patient was found to have one maternally derived, normal length allele of 17 CAG repeats and an expanded length allele of 45 CAG repeats, suggesting a paternal origin of expansion (Fig. 1). The patient's father was found to have alleles of 19 and 30 CAG repeats. Paternity was confirmed using eight polymorphic STR markers [Alford et al., 1994]. The probability of paternity was determined to be 99.92% (data not shown).

To clarify the origin of the expansion, linkage analysis was performed using PCR amplification of dinucleotide repeats flanking the *huntingtin* CAG repeat at D4S127 and D4S126 [Baxendale et al., 1993; Beutow et al., 1991; HD Collaborative Group, 1993; Lin et al., 1991; Richards et al., 1991; Squitieri et al., 1994; Tagle et al., 1993; Taylor et al., 1992; Zuo et al., 1993] (Fig. 2). These data confirmed the paternal origin of the CAG repeat expansion in our patient and indicated that the expansion arose from the chromosome carrying 30 CAG repeats.

PCR Analysis of Repeat Length at the HD Locus

Initial reports on the discovery of the *huntingtin* gene described a PCR assay that included an adjacent, downstream, polymorphic CCG repeat. It was found that while most HD alleles carry 7 tandem CCG repeats, the CCG repeat number can vary from 7 to 12 repeats [Andrew et al., 1994]. In our patient population, we have found, similar to Andrew et al. [1994], that 94% of HD length alleles and 57% of normal length alleles carried 7 CCG repeats (Fig. 3).

DISCUSSION

Genetic Counseling of Sporadic HD Families

A number of complex genetic and psychosocial issues can arise when providing DNA testing and genetic counseling services to individuals at risk for HD. These issues can include the psychological and sociological impact of the results of predictive testing on an individual and their family, and concerns by the patient for loss of employment or insurance and for recurrence in

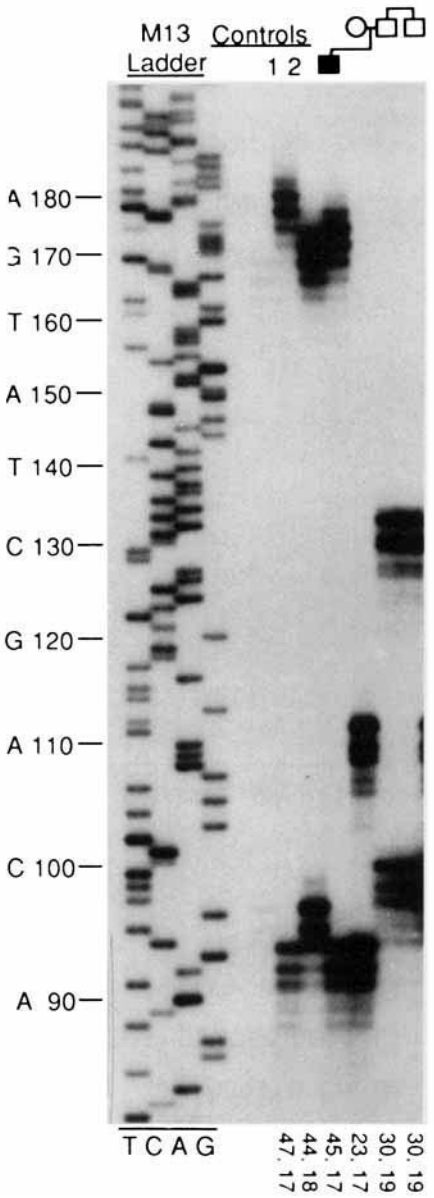

Fig. 1. PCR amplification of HD CAG repeats. PCR analysis of CAG repeat length in the family of a sporadic HD patient. HD allele sizes were determined by comparison to controls of known length and to an M13 ladder. The M13 ladder is labeled here by nucleotide number, HD controls are shown, and an abbreviated pedigree is given. HD allele sizes shown below the photograph are given in number of tandem triplet repeat units.

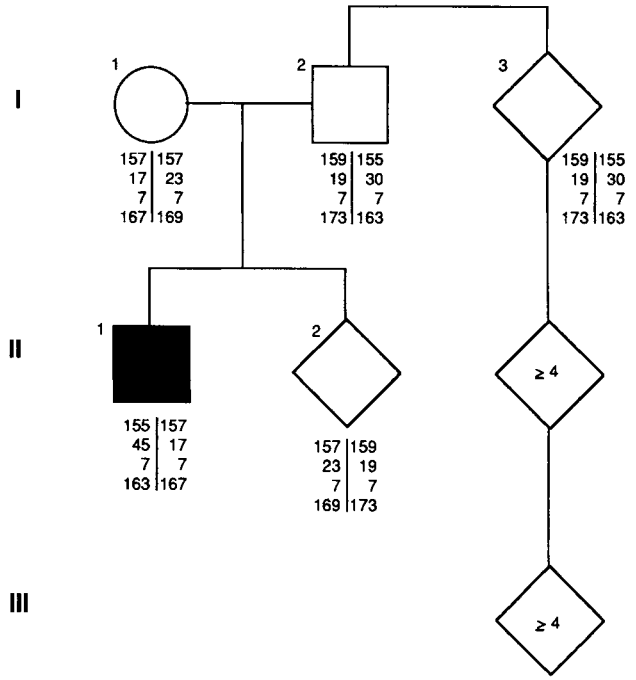

Fig. 2. Pedigree. Abbreviated pedigree for the family of this sporadic HD patient. The pedigree is presented in abbreviated form to protect the anonymity of the family. Individual II-1 is the proband. Allele sizes for all markers are shown for all individuals tested. Individuals not tested were not available. Allele sizes for linked markers D4S127 and D4S126 are given in base pair lengths. Allele sizes for intragenic HD repeats HD CAG and HD CCG are given in number of tandem triplet repeat units. Markers are shown in order from top to bottom: D4S127, HD CAG repeats, HD CCG repeats, D4S126.

future generations. Caution is taken during testing and counseling to assure resolution of the individualized issues that may arise with each case. Recently, a number of issues frequently encountered in HD testing and counseling have been considered in detail and a set of recommendations presented for testing and counseling of HD [Benjamin et al., 1994; Broholm et al., 1994; Hersch et al., 1994].

In particular, the identification of an intermediate allele (IA) presents a very complex genetic counseling situation due to the uncertainty of prognosis for patients carrying large IAs and the inability to predict the risk of HD for future generations. Smaller IAs, between 29 to 35 repeats, appear not to be associated with disease; however larger IAs, between 36 to 38 repeats have been shown, in some cases, to be associated with HD [Andrew et al., 1993; Duyao et al., 1993; Goldberg et al., 1995; Hersch et al., 1994; Myers et al., 1993; Telenius et al., 1995]. In addition, the risk to future generations for inheritance of an affected length allele from a parent with an IA cannot be reliably determined at this time [Benjamin et al., 1994; Broholm et al., 1994; De Rooij et al., 1993; Goldberg et al., 1993a,b,c, 1995; Hersch et al., 1994; Kremer et al., 1995; Myers et al., 1993; Telenius et al., 1994, 1995]. In our experience, sporadic cases of HD, caused by expansion of unstable intermediate alleles (IAs) to full mutations, represent the most challenging diagnostic and counseling situations for HD because the mechanism and frequency of new mutations at this locus is not clear at this time and risk for extended family members cannot be determined [Benjamin et al., 1994; Broholm et al., 1994; De Rooij et al., 1993; Goldberg et al., 1993a,b,c, 1995; Hersch et al., 1994; Kremer et al., 1995; Myers et al., 1993; Telenius et al., 1994, 1995].

Little is known about the mechanism or frequency of CAG repeat expansion and contraction for intermediate length alleles at this locus. Recent studies suggest that intergenerational changes in repeat length of nor-

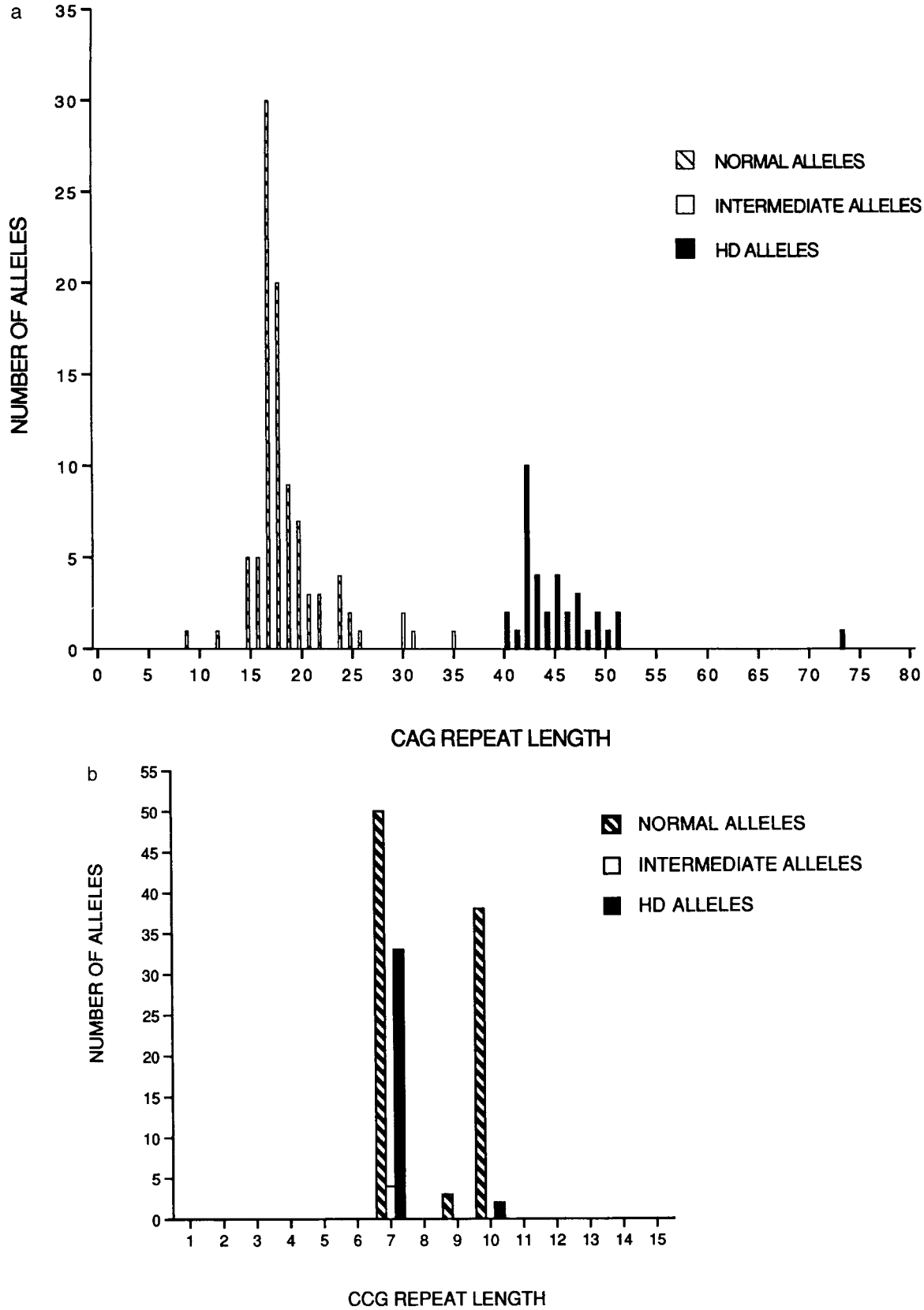

Fig. 3. CAG, CCG allele frequency, and distribution. Ninety-five normal and thirty-five HD chromosomes were analyzed for CAG (**a**) and CCG (**b**) repeat length. The occurrence of repeat length alleles is shown on the Y-axis and distribution of allele sizes is shown on the X-axis. As shown here, normal alleles range in size from 8 to 28 CAG repeats, intermediate alleles from 29 to 38 CAG repeats and HD alleles from 39 or more CAG repeats [De Rooij et al., 1993; Goldberg et al., 1993b, 1995; Myers et al., 1993].

mal sized alleles is rare; however, instability of IAs during paternal and maternal transmission has not been thoroughly studied [Goldberg et al., 1993a, 1995; Kremer et al., 1995; Telenius et al., 1994, 1995]. Sporadic cases of HD have occurred most frequently as a result of transmission of an IA through the male germline, similar to this case report [Goldberg et al., 1993b; Kremer et al., 1995; Myers et al., 1993]. CAG repeat instability, represented by somatic and gonadal mosaicism for repeat length alleles, has been clearly demonstrated in individuals carrying affected length alleles [Telenius et al., 1994, 1995]. Somatic mosaicism in carriers of IAs has not been specifically reported although gonadal mosaicism has been described in individuals carrying IAs [Goldberg et al., 1995; Telenius et al., 1995]. Additional analyses on single sperm may help to determine whether the level of gonadal mosaicism for a given IA length allele is predictable enough to allow the development of standards for risk assessment in families of sporadic HD patients [Goldberg et al., 1995; Telenius et al., 1994, 1995].

The molecular mechanisms leading to de novo trinucleotide repeat expansion are not well understood. The linkage data shown here provide no evidence of recombination between flanking markers D4S126 and D4S127. If unequal crossing over had been responsible for this mutational event, a double crossover would be necessary to maintain the haplotype. Given the less than 1 cM distance (less than 1 Mb) between the markers, a double crossover event is extremely unlikely [Baxendale et al., 1993; Beutow et al., 1991; HD Collaborative Group, 1993; Lin et al., 1991; Richards et al., 1991; Squitieri et al., 1994; Zuo et al., 1993]. These data demonstrate that, similar to other unstable trinucleotide repeats [Mahtani and Willard, 1993], unequal crossing over during meiosis is an unlikely mechanism for de novo expansion of the repeat. Alternatively, unequal gene conversion or sister chromatid exchange could explain the expansion without recombination of the flanking markers. In myotonic dystrophy, another triplet repeat expansion disease, a gene conversion event has been proposed to explain contraction of a repeat during transmission [O'Hoy et al., 1993].

Both cis-acting and trans-acting elements may influence stability of the HD CAG repeat, but no such factors have been identified. Recent evidence indicates that certain male specific factors may play a role as HD alleles appear to be more unstable during transmission through the male germline than during transmission through the female germline [De Rooij et al., 1993; Goldberg et al., 1993a,b; Kremer et al., 1995; Myers et al., 1993; Telenius et al., 1994, 1995]. Further investigation into the mechanisms of trinucleotide repeat expansion may provide evidence of factors which participate in stability or instability of the HD CAG repeat.

As noted, identification of a sporadic case of HD or an IA in a family presents a complex genetic counseling dilemma as caution must be taken to avoid over interpretation of the results [Andrew et al., 1993; Benjamin et al., 1994; Broholm et al., 1994; Goldberg et al., 1993a, 1995; Hersch et al., 1994; Telenius et al., 1995]. This case represents a large family and the uncle of the proband (individual I-3) has a number of children and grandchildren whose concern for inheritance of an IA by asymptomatic relatives could cause considerable anxiety for these and future generations (Fig. 2). Since little is known about the mitotic or meiotic instability of IAs or about the somatic or gonadal mosaicism of individuals carrying IAs, recurrence risk cannot be accurately determined for the extended families of sporadic HD patients and genetic counseling of the families of sporadic HD patients may be complicated by the uncertainty [Benjamin et al., 1994; Broholm et al., 1994; De Rooij et al., 1993; Goldberg et al., 1993a,b,c, 1995; Hersch et al., 1994; Kremer et al., 1995; Myers et al., 1993; Telenius et al., 1994, 1995]. Analysis of repeat length stability in the somatic tissues of individuals carrying IAs may provide valuable information about the mitotic stability of IAs and allow for the assessment of risk in IA carriers. Analysis of IA repeat length distributions in sperm may provide valuable information about intergenerational stability of IAs. Studies such as these may allow for the development of diagnostic standards for risk assessment in both present and future generations of families of sporadic HD patients [Goldberg et al., 1993a,c, 1995; MacDonald et al., 1993; Telenius et al., 1994, 1995]. Currently, we make concerned families of sporadic patients aware of testing and counseling programs that would be available to successive generations for assessing size and stability of IAs across generations [Benjamin et al., 1994; Broholm et al., 1994; Hersch et al., 1994].

Laboratory Testing for HD

Given the psychosocial impact of a diagnosis of HD on a family, accuracy in CAG repeat length estimation is crucial. Two factors play an important role: the sizing accuracy and the assay method. Accuracy and precision in sizing of alleles is essential to the detection and diagnosis of HD. We routinely use M13 sizing ladders, positive controls of known repeat length (Fig. 1), and require at least two independent reviews of the data. We have found routine proficiency testing for diagnostic reference laboratories to be a useful tool for assessing and improving inter- and intra-laboratory variations in size estimation.

There are currently available three PCR assays for determination of CAG and CCG repeat lengths in this region of the *huntingtin* gene. Most repeat size estimations using the CAG+CCG assay assume seven CCG repeats; however, inclusion of the variable length CCG in the PCR assay may, in some cases, result in a slight overestimation of CAG repeat size [Andrew et al., 1994]. Overestimation of repeat length becomes a crucial issue when alleles fall in the borderline ranges for normal, intermediate, and affected lengths. In our experience, the use of PCR primers that exclude the CCG repeat for amplification of this region provide a greater level of accuracy in allele sizing and eliminates the possibility for confusing results when testing multiple family members.

CONCLUSIONS

A combination of PCR assays for the CAG repeat and an adjacent variable length CCG repeat in the

huntingtin gene can increase the accuracy of molecular diagnosis for HD and in some cases, clarify ambiguous results. Nevertheless, a particularly difficult counseling issue arises for the families of sporadic HD patients. Risk assessment for these families is not possible at this time. Additional information about the intergenerational stability of intermediate length CAG repeat alleles will be required before useful guidelines for counseling these families can be developed.

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